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Short-Range and Long-Range Guidance by Slit and Its Robo Receptors: A Combinatorial Code of Robo Receptors Controls Lateral Position

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Summary

Slit is secreted by midline glia in *Drosophila* and functions as a short-range repellent to control midline crossing. Although most Slit stays near the midline, some diffuses laterally, functioning as a long-range chemorepellent. Here we show that a combinatorial code of Robo receptors controls lateral position in the CNS by responding to this presumptive Slit gradient. Medial axons express only Robo, intermediate axons express Robo3 and Robo, while lateral axons express Robo2, Robo3, and Robo. Removal of *robo2* or *robo3* causes lateral axons to extend medially; ectopic expression of Robo2 or Robo3 on medial axons drives them laterally. Precise topography of longitudinal pathways appears to be controlled by a combination of long-range guidance (the Robo code determining region) and short-range guidance (discrete local cues determining specific location within a region).

Introduction

Specificity of central nervous system (CNS) wiring unfolds during development as growth cones make a sequential series of cell-specific decisions about where to project. Early in their navigation, a major choice point confronting most CNS growth cones is the decision of whether or not to cross the midline. In bilaterally symmetric nervous systems such as the insect nerve cord or vertebrate spinal cord, a majority of CNS axons cross the midline once (and never do so again), while a minority never cross the midline and project on their own side. How they make this initial decision has been the subject of intense investigation for the past decade (e.g., Seeger et al., 1993; Serafini et al., 1994).

Repulsion plays a powerful role in controlling this decision. The Robo receptor is a key component of this process (Kidd et al., 1998a, 1998b) and Slit is the midline repellent that functions as the Robo ligand (Brose et al., 1999; Kidd et al., 1999). Robo on its own cannot explain all Slit function in controlling midline guidance. In our related paper (Simpson et al., 2000 [December issue of *Neuron*]), we described the family of three Robo receptors in *Drosophila*, and examined the unique and combinatorial roles of Robo and Robo2 in shaping the behavior of growth cones at the midline. A companion paper from our colleagues in the Dickson laboratory (Rajagopalan et al., 2000a [December issue of *Neuron*]) presents similar results.

Here we consider the next step in the process of CNS axon guidance: the choice of longitudinal pathway. Having decided whether or not to cross the midline, growth cones that project within the CNS next choose a specific longitudinal pathway. We show here that Slit and its family of Robo receptors play key roles in the control of this guidance decision as well.

Studies in the early 1980s on the developing CNS of the grasshopper embryo revealed a remarkable degree of specificity in the ability of individual growth cones to choose specific longitudinal axon pathways (Raper et al., 1983a, 1983b, 1983c, 1984; Bastiani et al., 1984). The growth cones from a group of six related neurons (Q1, Q2, G, C, Q5, and Q6) all extend together toward and across the midline. Once they cross the midline, their behavior dramatically changes as each growth cone makes a cell-specific decision about which longitudinal axon pathway to follow. Q1 and Q2 turn posteriorly in the medial MP1 pathway, G turns anteriorly in the lateral A/P pathway, C turns posteriorly in a neighboring lateral pathway, and Q5 and Q6 turn anteriorly in a specific intermediate pathway.

EM analysis revealed that the cell-specific turning is preceded by extensive filopodial contact with specific axons. For example, the filopodia of the G growth cone contact the initial four axons in the lateral A/P fascicle (two P and two A axons) and many other axon pathways, but display a specific affinity for the two P axons. G turns anteriorly along the A/P fascicle, selectively fasciculating with the P axons.

When the two A neurons were selectively ablated, G still turned anteriorly along the P axons. When the two P neurons were selectively ablated, the G growth cone did not choose a longitudinal pathway, but instead branched abnormally in the lateral neuropil, displayed no clear affinity for any particular longitudinal pathway, and sometimes continued to extend laterally to exit the neuropil.

These experiments demonstrated a remarkable degree of specificity of the G growth cone for the P axons (as compared to over 100 axons in ~20 different pathways). These results gave rise to the labeled pathways hypothesis (Raper et al., 1983b, 1983c; Goodman et al., 1984), which proposes that axon pathways are differentially labeled, and that follower growth cones are differentially determined in their ability to make specific choices of which labeled pathway to follow.

The G growth cone extends right past the same pathway (A/P fascicle) on its own side of the midline and shows no interest in it, but once it crosses the midline, G displays a specific affinity for the P axons on the other side. This change in behavior after crossing the midline suggested that the expression of specific receptors on growth cones must be a dynamic process (Goodman et al., 1985).

Monoclonal antibody screens were used to identify, purify, and characterize candidates for axonal recogni-

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tion molecules (Bastiani et al., 1987; Patel et al., 1987; Harrelson and Goodman, 1988; Zinn et al., 1988; Kolodkin et al., 1992). Other methods were used as well to identify additional pathway labels (e.g., Nose et al., 1992). Most of these surface labels were shown to function as homophilic cell adhesion molecules (e.g., Harrelson and Goodman, 1988; Snow et al., 1989; Elkins et al., 1990; Nose et al., 1992). One pathway label was a transmembrane Semaphorin (Kolodkin et al., 1992; Kolodkin et al., 1993) and was shown to function as both an attractant and a repellent (Wong et al., 1997; Yu et al., 1998). These pathway labels were found to be expressed on longitudinal segments but not on commissural segments of the same axon (Bastiani et al., 1987); their surface expression changes dynamically as axons cross the midline.

Of these pathway labels, the one best understood functionally is *Drosophila* Fasciclin II (Fas II), a cell adhesion molecule expressed on a subset of longitudinal axon pathways. Fas II is normally expressed on four major longitudinal axon pathways (out of a total of 20 or more), of which three are clearly visible in a single optical focal plane and are used widely as a diagnostic for CNS pathway selection. One of the Fas II pathways (the pCC pathway) is medial, another is intermediate (the MP1 pathway), and a third is lateral. Genetic analysis of *FasII* function revealed complementary loss-of-function and gain-of-function phenotypes (Lin et al., 1994). *FasII* loss-of-function led to a complete or partial defasciculation of all three major Fas II pathways. Driving specific Fas II expression in transgenic experiments led to rescue of the loss-of-function phenotype. Moreover, the gain-of-function can alter fasciculation by abnormally fusing the medial and intermediate Fas II pathways together (by preventing their pioneer axons from defasciculating at a specific choice point). These results show that Fas II indeed functions as a pathway label to control selective axon fasciculation.

The genetic analysis of Fas II function confirmed its role as a pathway label, but at the same time led to a puzzle. Fas II and all of the known pathway labels are expressed on several different longitudinal pathways that are spatially distinct. While Fas II is required by a growth cone to extend in one of the three major Fas II bundles, this analysis left unsolved the question of how this growth cone distinguishes one Fas II pathway from another. The 1994 Fas II paper (Lin et al., 1994) posed the question:

> "Are different Fas II-positive pathways distinguished by combinatorial labeling in which Fas II is necessary but not sufficient? . . . The results suggest that, though Fas II functions to control specific patterns of selective fasciculation, Fas II on its own cannot be the sole determinant of whether any particular growth cone does or does not fasciculate with any specific axon pathway. Rather, it appears to function in the context of other synergistic and competing guidance forces."

What are those other synergistic or competing forces? In the present paper, we present data that helps solve this puzzle. Discrete short-range guidance cues provided by pathway labels such as Fas II are only part of the story. Growth cones respond to a repulsive gradient of Slit emanating from the midline. Long-range and short-range cues together specify precise lateral position.

We use genetic analysis to show that a combinatorial code of Robo receptors controls lateral position in the CNS by responding to this Slit gradient. Medial axons express only Robo, intermediate axons express Robo3 and Robo, and lateral axons express Robo2, Robo3, and Robo. Removal of *robo2* and/or *robo3* causes lateral axons to extend medially. The lateral Fas II pathway fuses with the intermediate one, or the intermediate Fas II pathway fuses with the medial one. Ectopic expression of Robo2 or Robo3 on medial axons causes them to extend laterally. Ectopic Robo2 expression drives medial axons further laterally than does ectopic Robo3 expression. Ectopic Robo2 expression drives different medial axons to different lateral positions in a cell-specific fashion. The axons of the dMP2 and vMP2 neurons, which express Fas II and normally extend in the medial Fas II pathway, are driven to extend in the intermediate Fas II pathway (and sometimes the lateral Fas II pathway) when they express Robo2.

We propose a model whereby the precise topography of longitudinal pathway choices is controlled by a combination of long-range and short-range guidance. Regional specification is determined by the Robo code in response to the Slit gradient. Within a region, the specific choice of pathway is determined by local cues, such as Fas II and other pathway labels. In a companion paper, our colleagues in the Dickson laboratory report on similar discoveries of the Robo code and its role in controlling lateral position (Rajagopalan et al., 2000b [this issue of *Cell*]).

Results

Differential Expression of Robo Family Members

In our related paper (Simpson et al., 2000), we described the identification of three Robo family members in *Drosophila*. Antibodies against Robo, Robo2, and Robo3 were generated in mice and used to examine the protein localization of the three Robos in the *Drosophila* embryonic CNS.

As described in Simpson et al. (2000), in situ hybridization using RNA probes and immunocytochemistry using antibodies show that all three Robos are expressed in the embryonic CNS during the period of axon outgrowth. *robo* expression begins first at embryonic stage 10. *robo2* expression is first visible at stage 11 and becomes restricted to a smaller subset of neurons later in development by stage 15. *robo3* expression does not begin until late stage 13, and is limited to fewer neurons.

Robo and Robo2 together play an early function in the control of midline crossing. *robo* continues to be expressed by all neurons, and Robo protein appears at high levels on axons either after they cross the midline, or from the outset if they never cross the midline. Robo2 is more dynamic in its pattern of expression. Initially, it is expressed by a wide range of neurons, including all of the early pioneer neurons whose axons do not cross the midline (e.g., pCC, MP1, dMP2, and vMP2). But during the period around late stage 13 in which these axons

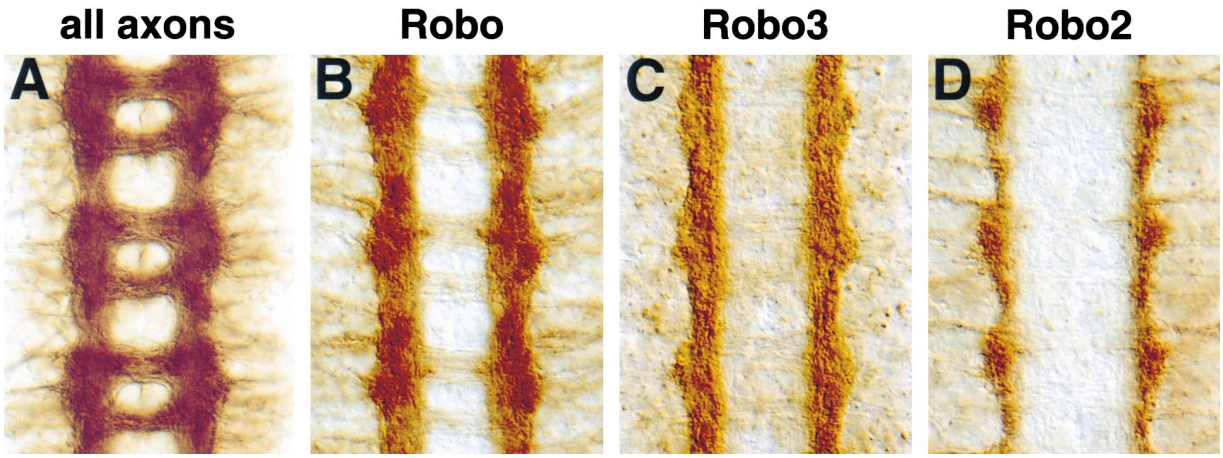

Figure 1. The Robo Receptor Protein Family Members Have Distinct Patterns of Expression

(A) Wild-type stage 16 *Drosophila* embryos stained with the pan-neural antibody (mAb BP102) to reveal the ladder-like scaffold of axon pathways in the CNS. There are thick, longitudinal connectives running up and down each side of the midline; these connectives are made up of ~20 or more bundles of axons arranged from medial to lateral. Each segment has two major commissures that cross the midline.
(B)–(D) show the expression patterns of Robo, Robo3, and Robo2 in wild-type embryos in dark brown. Anti-Robo (mAb 13C9) (B) reveals Robo protein on the whole width of the longitudinal tracts, but not on the commissural tracts. Anti-Robo3 (mAb 14C9) (C) reveals Robo3 on the outer two-thirds of the longitudinal tracts. A polyclonal antibody to Robo2 (D) shows that the high level of Robo2 expression is restricted to the lateral third of the longitudinal tracts farthest from the midline.

selectively defasciculate to form the medial pCC pathway and the intermediate MP1 pathway, the expression of Robo2 declines in many of these neurons. It is during this same period (late stage 13 to stage 14) that Robo3 begins to be expressed by a subset of neurons.

From stage 14 onward, as multiple longitudinal pathways form, all three Robos are expressed on some or all longitudinal axon tracts and are excluded from commissural axon tracts (Figure 1). Within the longitudinal tracts, their expression patterns differ dramatically. Robo is found on all longitudinal axon pathways (Figure 1B). The second phase of Robo2 expression, and the only phase of Robo3 expression, have a common quality. Both are expressed on a subset of axons that extend in specific lateral positions of the developing CNS. Robo3 is expressed only on axons that extend in the outer two-thirds of the longitudinal pathways (the intermediate and lateral regions) (Figure 1C). A high level of Robo2 expression is restricted to axons that extend in the outer third of the longitudinal pathways (the lateral region), farthest from the midline and thus farthest from the source of Slit (Figure 1D).

All three Robos show relatively tight boundaries. All three are absent from the commissures, and Robo3 and Robo2 are restricted to certain regions of the longitudinal pathways. The expression of Robo3 and Robo2 is not graded, but rather appears to form regional boundaries. While the high level of Robo2 is restricted to the lateral pathways, we also detect a lower level of Robo2 expression on some of the intermediate pathways (the more lateral ones). This is most easily visualized using further amplification steps in the immunocytochemistry. The low level of Robo2 expression begins right in the middle of the intermediate Fas II pathway. This step-wise expression of Robo2 (from none on the medial portion of the intermediate pathways, to a low level on the lateral portion of the intermediate pathways, to a high level laterally) reveals further regional subdivisions of the longitudinal pathways.

The pattern of expression of Robo3 and Robo2 in individual identified neurons is consistent with their overall patterns of expression. For example, *robo3* RNA is expressed in the MP1 neuron whose axon pioneers the intermediate Fas II pathway. *robo3* is largely absent from pCC and other neurons whose axons pioneer the medial Fas II pathway. All of these neurons (e.g., MP1, pCC) transiently express *robo2* when they are making the earlier decision not to cross the midline, but that expression declines by the time the medial and intermediate Fas II longitudinal pathways separate from one another. This is consistent with the presence of Robo2 only on lateral axons during later stages of development.

Immunoelectron microscopy using antibodies against the three Robos reveals further features of their patterns of expression (Figures 2A–2C). In cross sections of the nerve cord at stage 16, we can identify around 150 axons organized into 15–20 distinct bundles. The immuno-EM reveals staining in the same regions in which we detect high levels of expression at the light level. All three Robos are expressed on specific growth cones and filopodia. Commissural growth cones and axons are devoid of all three Robos. All ~150 longitudinal axons express Robo. The ~50 intermediate axons and the ~50 lateral axons express Robo3 (with one exception; see below). The ~50 lateral axons express Robo2. Most lateral axons express Robo2 and Robo3, with one exception; the most lateral axon bundle of ~10 axons expresses high levels of Robo2, but is largely or completely devoid of Robo3 (Figure 2B).

When we compare immuno-EM sections stained with the three different Robos with similarly staged sections stained with anti-Fas II, we find a consistent pattern (summarized in Figure 2D). The medial Fas II pathway (~18 axons) is in the medial region where only Robo is

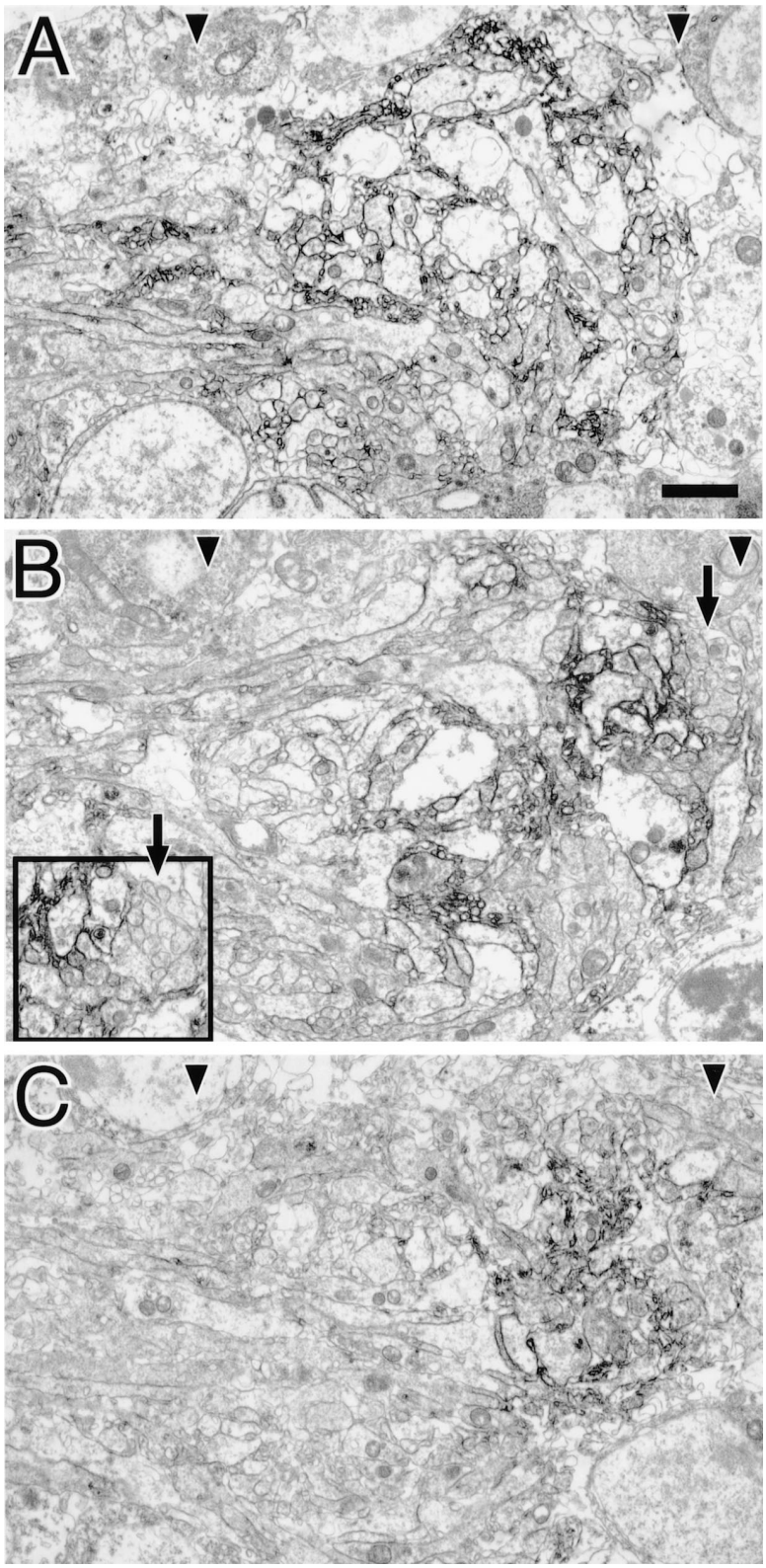

Figure 2. Immunoelectron Microscopy of Robo, Robo2, and Robo3 Protein Expression in the Embryonic Central Nervous Systems

Immunohistochemistry using antibodies to the Robo proteins was performed and the stained embryos were sectioned perpendicular to the axon scaffold for electron microscopy. The micrographs shown are cross-sections of wild-type stage 16 embryos at the level of the posterior commissure. The right longitudinal tracts from segments in the abdominal region are shown. The black arrowheads denote the medial (left) and lateral (right) edges of the longitudinal tracts. The beginnings of the commissural tracts are visible on the left side of each micrograph. The dark precipitate indicates the membranes of cellular processes expressing the Robo proteins; the darkest stain are the small profiles of filopodia and the larger profiles of growth cones. Robo, Robo2, and Robo3 staining is seen around axons, but is concentrated on growth cones and in particular on small filopodial processes. In (A), Robo protein is present on axons throughout the entire width of the longitudinal tract. Robo3 expression is shown in (B), and only axons in the lateral two-thirds of the longitudinal show the dark labeling. There is a group of axons in the dorsal, lateral-most part of the longitudinal tracts that does not stain with Robo3. The lack of expression of Robo3 on this one lateral-most bundle is consistent from segment to segment and embryo to embryo. The inset in (B) is another example showing the absence of Robo3 on this bundle of axons. These axons express Robo and Robo2, but not Robo3. (C) shows Robo2 expression in the lateral third of the longitudinal tract. Scale bar = 1 μm. The schematic diagram in (D, facing page) shows the position of the domains of expression of the Robo receptors in a cross section of the developing nerve cord (in correspondence with the electron micrographs in [A]–[C]). The schematic also shows the major Fasciclin II expressing bundles relative to the regions of expression of each of the Robo receptors. The medial (M), intermediate (I), and lateral (L) Fas II-positive axon fascicles are labeled. The lateral bundle of axons that express Robo and Robo2 but not Robo3 (corresponding to the inset in [B]) are shown in dark yellow at the lateral-most part of the longitudinal bundle. Robo2 appears to be expressed in two steps. It is expressed at a low level in the lateral half of the medial Robo3-positive region; this staining is revealed at the light level using amplification protocols. Robo2 is expressed at a much higher level in the lateral region.

expressed. The intermediate Fas II pathway (~10 axons) is in the intermediate region where Robo3 is also expressed. At the light level, the intermediate Fas II pathway appears to be right at the boundary between no Robo2 expression and a low level of Robo2 expression. The lateral Fas II pathway (~8 axons) is in the lateral region where Robo2 and Robo3 (as well as Robo) are expressed at high levels.

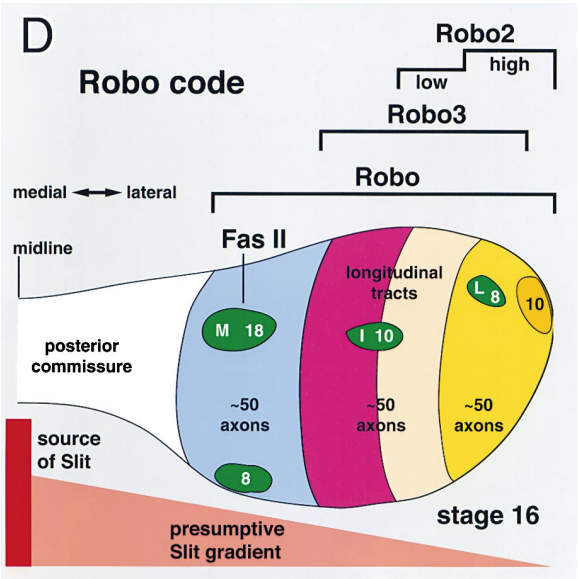

Figure 2. (continued).

Loss-of-Function Analysis of Robo Family Members

The mutant phenotypes of *robo*, *robo2*, and *robo3* support the hypothesis that the Robos specify lateral position with respect to the midline. *robo* mutants show axons ectopically crossing and recrossing the midline (Seeger et al., 1993; Kidd et al., 1998a, 1998b). These axons are predominantly those of the innermost part of the longitudinal scaffold. When a *robo* mutant is examined with anti-Fas II (mAb 1D4), only the medial Fas II pathway crosses the midline (Figures 3 and 4). The intermediate and lateral Fas II pathways stay on their own side. One possible interpretation is that the intermediate and lateral expression of Robo3 and Robo2 keeps these axons from crossing the midline in a *robo* mutant. In Simpson et al. (2000), we showed that in a *robo*, *robo2* double mutant, all axons go to the midline and do not leave it (and thus look like a *slit* mutant).

robo2 loss-of-function mutations show occasional ectopic midline crossing, but, more prominently, they show abnormalities in lateral positioning (Figures 3C and 4). The most common phenotype as revealed with anti-Fas II staining is crossovers and "braiding" between the intermediate and lateral Fas II pathways (and sometimes between the medial and intermediate Fas II pathways). Although superficially the axon scaffold looks relatively normal in a *robo2* mutant (when visualized with mAb BP102, which labels all axons), the lateral positions of the longitudinal pathways are altered in the absence of Robo2.

In Simpson et al. (2000), we showed that *robo* and *robo2* mutants have differential effects on the normal pair of Connectin (Conn) longitudinal pathways. Conn is normally expressed on a medial pathway (below or ventral to the medial Fas II pathway) and on a second lateral pathway (which normally lies between the intermediate and lateral Fas II pathways; Nose et al., 1992). In a *robo* mutant, the medial Conn pathway abnormally crosses the midline, while the lateral Conn pathway is relatively normal in its location (Simpson et al., 2000 and Kidd et al., 1998b). In a *robo2* mutant, the lateral Conn pathway fuses with the medial Conn pathway, and all of the Conn axons extend together in the medial region (reviewed in Figure 7A).

The role of Robo3 in lateral positioning was examined using RNA interference (RNAi). The validity and specificity of the RNAi technique for these phenotypes was confirmed by injecting double-stranded RNA for 1 kb regions of *robo* and *robo2* into wild-type early embryos. The Fas II staining pattern of injected embryos allowed to develop until stage 16 closely resembles that of the *robo* and *robo2* mutants generated by conventional genetic techniques (Figures 3B, 3C, and 4). The severity of the phenotypes can vary within an embryo, tending to be stronger near the posterior end where the dsRNA is injected, but in no case was the RNAi phenotype substantially more severe or qualitatively different from the genetic mutant. This confirms that RNAi is specific for the Robo family member gene to which it is targeted, and does not affect these closely related genes.

Injection of *robo3* dsRNA causes the stage 16 embryo to have two Fas II longitudinal pathways instead of three (Figures 3D and 4). The intermediate pathway is missing, and the medial pathway is larger than normal. The lateral (normally Robo2 expressing) Fas II pathway appears normal. In the absence of Robo3, the medial and intermediate Fas II pathways fail to separate, and the intermediate (normally Robo3 expressing) pathway does not properly form.

The *robo2, robo3* double mutant, generated by injecting *robo3* dsRNA into a *robo2* mutant, contains a large single Fas II longitudinal pathway (Figures 3G and 4). This single fascicle is thicker than wild-type Fas II pathways and is close to the midline in the normal location of the medial Fas II pathway. Sometimes we also see a small, more lateral Fas II bundle, but this is thinner and more variable than normal (Figure 3G). In the absence of both Robo3 and Robo2, it appears as if most (and in some cases all) of the Fas II axons selectively fasciculate into one Fas II pathway in the medial position (Figures 3G and 4). This suggests that Robo3 and Robo2 are required for the normal formation of the Robo3 expressing intermediate Fas II pathway and the Robo2/Robo3 expressing lateral Fas II pathway.

Unlike *robo* mutants alone, *robo, robo3* embryos (*robo* mutants with *robo3* dsRNA) have ectopic crossing of the intermediate Fas II pathway as well as the medial one (Figures 3F and 4). This resembles the addition of the two individual phenotypes: lack of Robo3 causes the intermediate pathway to join the medial pathway, and the lack of Robo allows this fused Fas II pathway to weave back and forth across the midline. In the *robo, robo3* double mutant, the outer Fas II pathway remains on its appropriate side, presumably due to the presence of Robo2.

These phenotypes support the model that Robo is the most important contributor to maintaining the Fas II pathways on the appropriate side of the midline, but that Robo2 and Robo3 determine the lateral position of these and other longitudinal pathways (reviewed in Figure 7A). Robo3 specifies the intermediate region and its pathways, while Robo2 specifies the lateral region and its pathways.

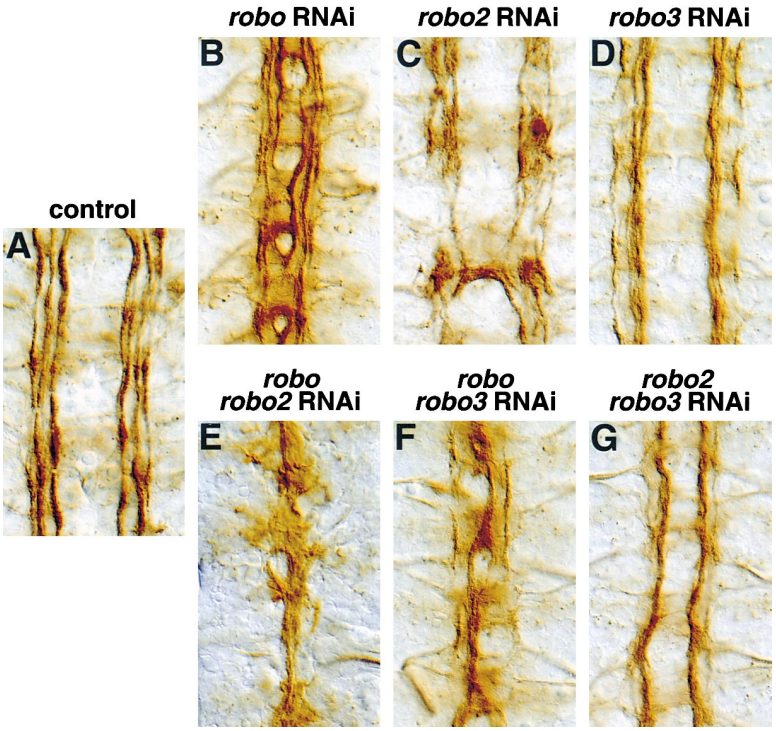

Figure 3. Mutant Phenotypes Alone and in Combination of Robo Family Members

(A) In embryos injected with buffer (as a control for the RNAi method) and stained with anti-Fasicilin II (1D4), three distinct longitudinal bundles are visible on each side of the midline. From the midline out, the bundles are called the medial, intermediate, and lateral Fas II pathways.

(B) The phenotype of embryos injected with *robo* dsRNA resembles that of a *robo* EMS point mutant: the medial Fas II bundle ectopically crosses the midline in every segment.

(C) In embryos injected with *robo2* dsRNA, we see the same phenotype as in *robo2* deletion mutations. We occasionally observe ectopic crossing of the midline, but the most common defects are braiding or altered positioning of the intermediate and lateral fascicles.

(D) *robo3* mutants generated by injection of dsRNA form only two Fas II-positive fascicles—the medial and the lateral. The intermediate Fas II fascicle fails to form because its axons fail to leave and join the medial Fas II fascicle.

(E) The *robo, robo2* double mutants, made either by recombination of two genetic mutants or by injecting *robo2* dsRNA into a *robo* mutant as shown here, have only a single Fas II fascicle running right along the midline. The entire CNS is collapsed onto the midline because the neurons that lack Robo and Robo2 signaling cannot detect the Slit repellent secreted from the midline.

(F) The *robo, robo3* mutant, made by injecting *robo3* dsRNA into a *robo* mutant, has two Fas II fascicles, the medial one, which is thicker than normal because it contains axons that are normally part of the intermediate Fas II fascicle, and the lateral Fas II fascicle. This combined medial and intermediate fascicle ectopically crosses and recrosses the midline, as the medial Fas II fascicle does in a *robo* mutant. The lateral Fas II fascicle remains ipsilateral because it still expresses Robo2.

(G) The *robo2, robo3* double mutant, made by injecting *robo3* dsRNA into a *robo2* mutant, has a single Fas II fascicle on each side of the midline. This fascicle is thicker than normal because it contains axons that normally form the intermediate and lateral Fas II fascicles. It does not cross the midline because it still expresses Robo.

Gain-of-Function Analysis of Robo Family Members: Pan-Neural Overexpression of Robo2 and Robo3

Overexpression of Robo2 supports the model that Robo2 levels contribute to the lateral position of axons

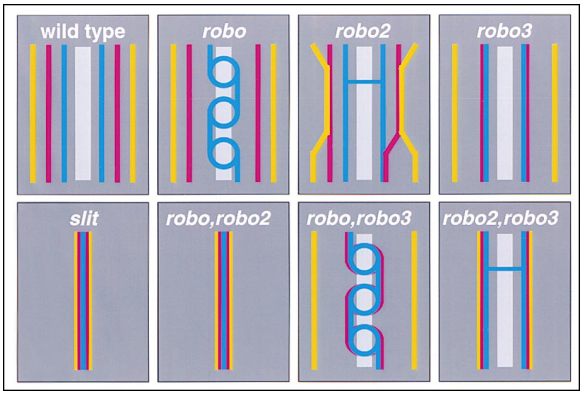

Figure 4. Schematic Diagram of Robo Family Mutant Phenotypes

This diagrammatic representation of the *robo* mutant phenotype combinations shows that Robo and Robo2 cooperate to govern midline crossing, while Robo2 and Robo3 together set the appropriate lateral positions of axons that form the three distinct Fas II fascicles. The medial Fas II fascicle expresses only Robo and is shown in blue. The intermediate Fas II fascicle in purple contains axons that express Robo and Robo3. The lateral Fas II fascicle in yellow is composed of axons expressing Robo, Robo2, and Robo3.

(Figure 5). Overexpression of *UAS-robo2* in all CNS axons using the *elav-GAL4* driver results in a commissureless-like phenotype (i.e., appearing like the *commissureless* mutant). There are a number of other genetic combinations that result in a commissureless-like phenotype. These all look the same when examined with mAb BP102 that stains all axons: the commissures are missing (see Simpson et al., 2000). But the appearance of the three Fas II pathways differs depending upon the genetic makeup of the embryo.

When Robo expression is increased on all axons, by either directly driving more Robo or in a *comm* mutant (in which Comm no longer downregulates Robo), we still detect three distinct Fas II pathways (Figure 5C). This is true even when the Robo Y-F "hyperactive" receptor is transgenically expressed on all axons (Bashaw et al., 2000) (Figure 5D). Under these various conditions, there is disorganization of longitudinal pathways, but we can generally identify the three Fas II pathways.

But when Robo2 is ectopically expressed on all axons, the lateral position of the pathways is disrupted, and as a result, all three Fas II pathways are bundled together into a single, thick tract (Figure 5E). Ectopic Robo2, but not ectopic Robo or loss of Comm, is sufficient to override the endogenous positional information that specifies the locations of the three Fas II pathways.

Overexpression of Robo3 in all CNS axons (using the *elav-GAL4* driver) results in a weakly commissureless-

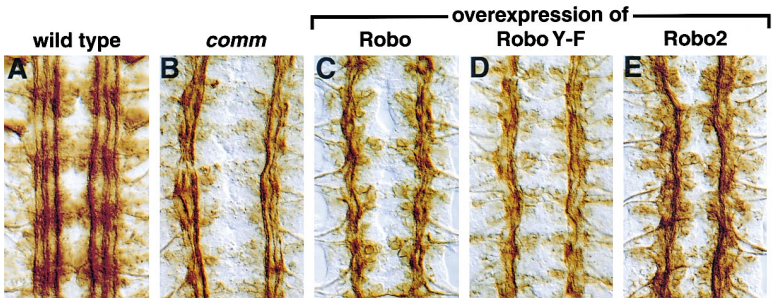

Figure 5. Pan-Neural Overexpression of Robo2

There are a number of ways to generate a commissureless-like phenotype where no axons can cross the midline. Loss of *commissureless* (B) and pan-neural overexpression of *robo* (C) or the hyperactive *robo Y-F* (D) all have no axons crossing the midline, but they usually retain three fascicles present on either side of the midline. Overexpression of *robo2* in all neurons (E) also generates a commissureless-like phenotype, but in this case, all three Fas II bundles are compressed into a single fascicle. Robo2 prevents axons that normally cross the midline from crossing it, but it also alters their ability to determine distinct lateral positions.

like phenotype. As with *UAS-robo* and *UAS-robo2*, the gain-of-function commissureless phenotype of Robo3 requires two copies of the *UAS-robo3* reporter to generate commissureless segments. Some of the Fas II bundles are fused, but at least two distinct fascicles are still visible at this level of overexpression (data not shown).

Gain-of-Function Analysis of Robo Family Members: Specific Ectopic Expression of Robo2 and Robo3 in Neurons with Medial Axons

Expressing Robo2 or Robo3 in subsets of neurons whose axons normally extend in medial longitudinal pathways can drive these axons to assume more lateral positions (Figures 6 and 7). Although both can drive medial axons further laterally, Robo2 and Robo3 are not identical: when tested on the same axons, Robo2 drives medial axons further laterally than does Robo3.

For example, in each abdominal hemisegment, three neurons express the transcription factor Apterous (Ap; Lundgren et al. 1995). These neurons normally extend their axons toward the midline, and then turn anteriorly on their own side close to the midline. These axons turn anteriorly in the medial region. When viewed at the light level with confocal microscopy, the Ap axons sometimes look like they are running just at the lateral edge of the medial Fas II bundle, and sometimes there is a little space between them (suggesting another axon or two interposed; this observation was confirmed by D. D. O'Keefe and J. B. Thomas, personal communication) (Figure 6). We use this staining pattern to infer that the Ap axons run in a medial axon pathway just lateral to the medial Fas II tract.

When these axons ectopically express Robo2 under control of *Apterous-GAL4* (*Ap-GAL4*; Calleja et al., 1996 and O'Keefe et al., 1998), they move laterally and extend anteriorly in a specific location between the intermediate and the lateral Fas II pathways (Figure 6). In fact, they extend in the medial-most region of the endogenous Robo2 expression zone (Figure 6I). The Ap axons from neighboring segments appear to pick the same lateral pathway, and to fasciculate together as they extend anteriorly from segment to segment (Figures 6G and 6H). We have tested two different GAL4 reporters (EP2582 inserted upstream of *robo2* and a *UAS-robo2* insert). Both drive different levels of Robo2 expression as indicated by their different strengths of pan-neural gain-of-function phenotypes. Nevertheless, both drive the Ap axons to the same lateral location. There is a second argument that supports this same conclusion. It is well known that the GAL4 expression system drives different levels of expression from a UAS transgene in different cells and segments of the same embryo. Yet, from cell to cell, segment to segment, and embryo to embryo, the Ap axons (expressing what we presume to be variable levels of Robo2) always turn anteriorly in the same lateral location between the intermediate and lateral Fas II pathways.

This suggests that Robo2 reading of the Slit gradient drives axons to a rough lateral position, regardless of the precise level of Robo2, after which local cues determine which specific pathway a particular axon joins. Thus, Robo2 drives the Apterous axons to the lateral third of the scaffold, and regardless of the precise level of Robo2, this is sufficient to allow their final pathway choice to be precisely and uniformly dictated by some unknown but specific local cue. All of our experiments are done at different Robo2 levels within a relatively narrow range. It is conceivable that much higher levels of Robo2, or Robo3, might drive axons further laterally, even into more lateral zones.

Using the same *Ap-GAL4* transgene to drive overexpression of Robo3 in the Ap axons leads to a different alteration in lateral position (Figures 6K, 6L, and 7). Whereas ectopic Robo2 drives these axons quite far laterally to a position between the intermediate and lateral Fas II pathways, the ectopic expression of Robo3 drives them to an intermediate position, just medial to the intermediate Fas II pathway (Figure 6K). Thus, Robo3 and Robo2 can both drive the medial Ap axons to more lateral positions, but they do so to different extents, as we might expect by their normal patterns of expression. Robo2 drives axons further laterally than does Robo3, relative to the intermediate Fas II bundle.

Using the same *Ap-GAL4* transgene to drive overexpression of either Robo or the hyperactive Robo Y-F (Bashaw et al., 2000) in the Ap axons leads to no alteration in lateral position (Figure 6). Even with more Robo, these axons still continue to extend in their normal medial location. These axons, and for that matter all longitudinal axons, normally express Robo, and we infer from this result that increasing the level of Robo does not alter the choice of lateral position by typical follower growth cones. The choice of lateral position by these axons is exquisitely sensitive to the presence of Robo2 and Robo3, but apparently not to the level of Robo.

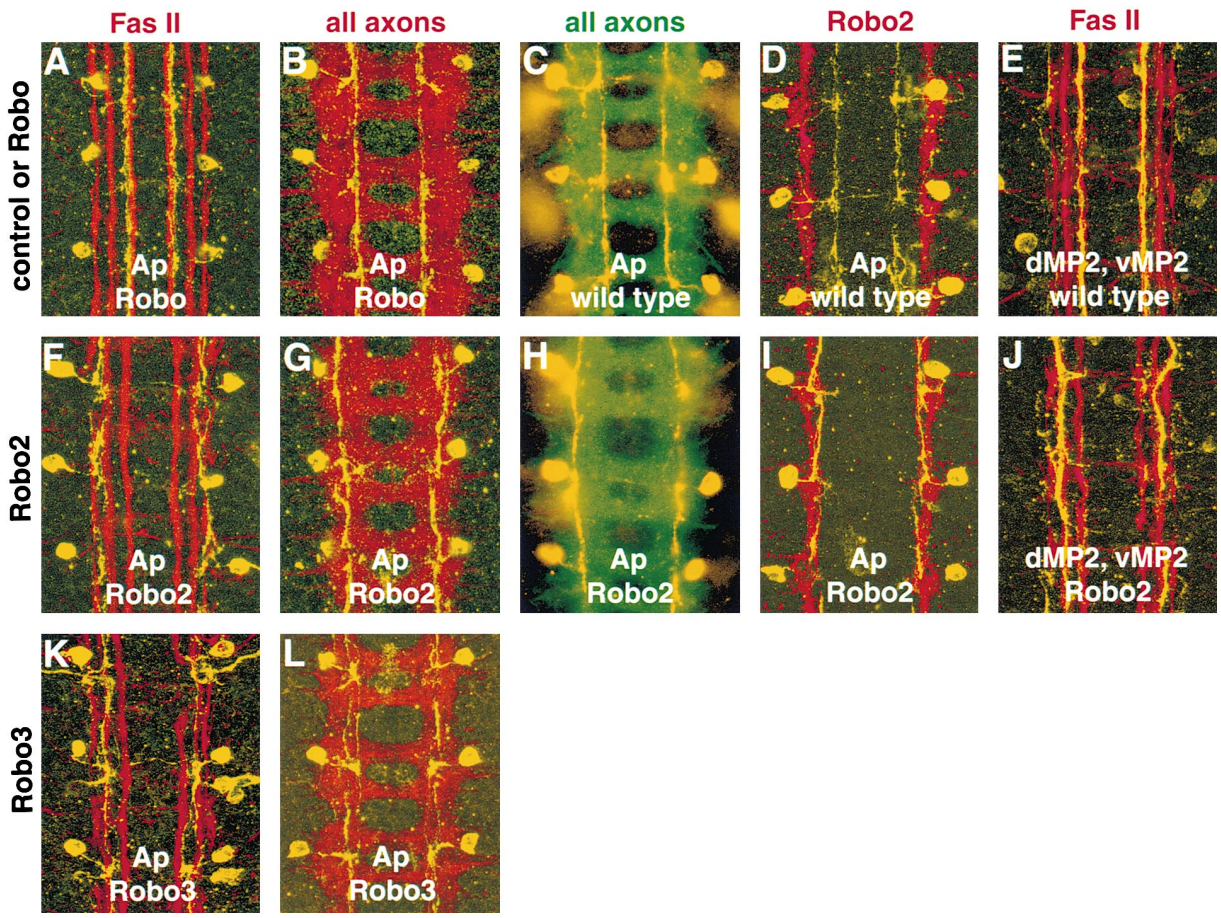

Figure 6. Overexpression of Robo2 on Specific Medial Axons Drives Them Lateral

(A–D, F–I, and K–L) The Apterous-expressing axons (Ap) normally extend toward the midline and turn to project anteriorly along the medial edge of the axon scaffold without crossing the midline. (A), (F), and (K) show Ap axons expressing *UAS-robo* (A), *EP-robo2* (F), and *UAS-robo3* (K) in yellow and their positions relative to the three major Fas II axon bundles (medial, intermediate, and lateral) in red. (B), (G), and (L) show the position of Apterous neurons stained with anti-βgal in yellow and the complete axon scaffold as stained with mAb BP102 in red. (B) The control experiment shows *Ap-GAL4 x UAS-robo*. (G) The experimental shows *Ap-GAL4 x UAS-lacZ; EProbo2.* Ectopic expression of *robo2* causes axons to lateralize. (L) shows *Ap-GAL4 x UAS-τlacZ*; *UAS-robo3*, in which axons move laterally, but not as far laterally as the do when they ectopically express *robo2.* Some Ap axons are still medial in this example. (C) shows Apterous axons expressing UAS-lacZ in yellow on a green axon scaffold visualized with a fluorescently conjugated anti-HRP. (H) Ectopically expressing Robo2 in the Apterous neurons drives their longitudinal projections (in yellow) farther from the midline, toward the lateral edge of the axon scaffold. (D) and (I) show the position of Apterous neurons in yellow relative to the staining of anti-Robo2 in red. When Robo2 is driven in the Ap neurons, they shift laterally to the edge of the normal Robo2 domain. (E) and (J) show overexpression of Robo and Robo2 using the *15J2-GAL4* line, which drives expression in the dMP2 and vMP2 neurons (and variably in a few other neurons; Hidalgo and Brand, 1997). The dMP2 and vMP2 axons normally extend in the medial Fas II pathway (just slightly medial to where the Ap axons extend). When Robo2 is driven in the dMP2 and vMP2 neurons, their axons shift laterally to extend in the intermediate Fas II pathway, and occasionally in the lateral Fas II pathway. All the images are confocal, except for (C) and (H), which are epifluorescence.

The ability of Robo2 to lateralize these axons does not depend on the presence of Robo: lateralization occurs in neurons that coexpress transgenic Robo2 and a Robo dominant negative receptor, or when Robo2 is expressed ectopically in a *robo* null mutant background.

We wanted to further analyze the role of the Robo code in determining lateral position, and the potential interplay of this long-range guidance system using the presumptive Slit gradient with other local cues. Are all medial axons driven to the same lateral positions by Robo3 and Robo2? Or, alternatively, are they driven to cell-specific lateral positions? If the latter is the case, are their other cues that help predict the specific location? To this end, we turned to other GAL4 lines to drive the expression of various Robo family members in other subsets of axons.

The *15J2-GAL4* line drives expression in the dMP2 and vMP2 neurons (and variably in a few other neurons; Hidalgo and Brand, 1997). These two neurons normally express Fas II, and normally extend in the medial Fas II pathway. Ectopic expression of Robo2 in these neurons leads to a bimodal phenotype (Figure 6J). The dMP2 and vMP2 axons always appear to extend in a Fas II pathway, but they now pick either the intermediate or lateral Fas II pathways. These axons are never found medially, are often found in the intermediate Fas II pathway, and occasionally are found in the lateral Fas II pathway. These are distinctly different locations than

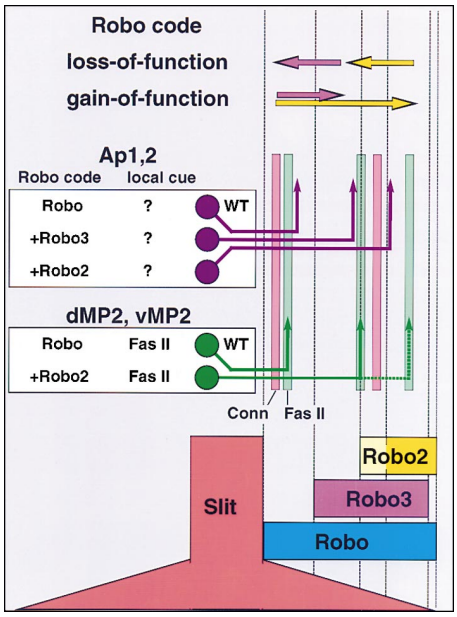

Figure 7. Schematic Diagram Showing the Role of the Robo Code and Local Cues in Determining Lateral Position

Bottom: Most of the Slit protein stays around the midline, while a small amount appears to diffuse away from the midline and form a gradient. Shown on the right are the regional patterns of expression of Robo, Robo3, and Robo2. Top: Loss-of-function of Robo2 and Robo3 drives lateral axons more medial (yellow and purple arrows, respectively), while gain-of-function of Robo2 and Robo3 (i.e., ectopic expression in axons that normally express only Robo and project medially) drives medial axons more lateral (yellow and purple arrows, respectively). Middle: Robo2 and Robo3 drive different axons to different locations. Precise lateral location is determined by a combination of the Robo code and local cues. The Ap axons, which project ipsilaterally, are driven to more lateral positions. The dMP2 and vMP2 axons are also driven to different lateral positions. They normally extend in the medial Fas II pathway. When they ectopically express Robo2, they are driven to extend in either the intermediate or occasionally in the lateral Fas II pathway. For implications, see Discussion.

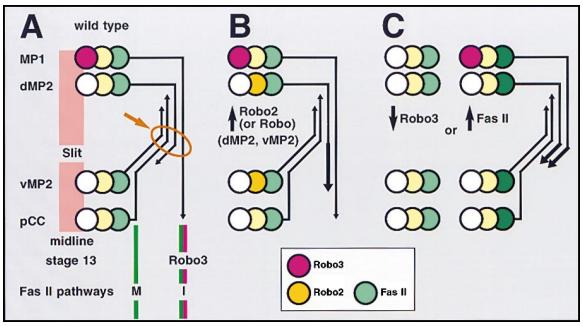

Figure 8. Schematic Diagram Showing Why Increased Robo Can Drive dMP2 and vMP2 to the Intermediate Fas II Pathway

For most axons, Robo3 and Robo2, but not Robo, control lateral position. However, there is one exception in which increased Robo can also alter lateral position. Of all of the GAL4 lines we have tested, the *15J2* line is the only line in which we can alter lateral position by increasing the expression of Robo. (A) Normally, the pCC growth cone pioneers the medial Fas II pathway (called the pCC pathway) as it extends anteriorly. The vMP2 growth cone fasciculates with it and follows right behind pCC. The MP1 growth pioneers the intermediate Fas II pathway (called the MP1 pathway) as it extends posteriorly. The dMP2 growth cone fasciculates with it and follows right behind MP1. In the middle of the segment, these four axons meet and transiently fasciculate together. These axons subsequently selectively defasciculate as pCC pioneers the medial Fas II pathway, while MP1 ultimately pioneers the intermediate Fas II pathway (this choice point is circled in brown and arrowed). vMP2 stays with pCC in the medial Fas II pathway. It appears as if dMP2 leaves MP1 and joins with pCC and vMP2 to join the medial pathway. The defasciculation of these axons, and their separation to form these two distinct longitudinal pathways, occurs during the time during which *robo2* expression in all of these neurons declines; this is the same period during which *robo3* appears in only MP1 of these four neurons. (B and C) It appears as if the choice by dMP2 and vMP2 of which axon to follow—pCC or MP1—is based on a delicate balance and is exquisitely sensitive to the levels of various Robo family members. (B) Increasing the level of Robo2 quite consistently tips the balance towards dMP2 and vMP2 extending more laterally with MP1 at the choice point. Increasing the level of Robo, on the other hand, has a more probabilistic effect on whether these growth cones stay with pCC (as normal) or go with MP1. This particular choice occurs at an early stage and in a confined lateral location. It appears to be much more sensitive to the levels of Robo than does the typical choice by follower growth cones of which preexisting longitudinal axon pathway to follow. (C) In contrast, decreasing the levels of Robo3 (in MP1), or increasing the levels of the cell adhesion molecule Fas II in all of these axons (Lin et al., 1994), prevents the MP1 and dMP2 axons from defasciculating with pCC and, as a result, they continue to extend in the medial Fas II pathway—the pCC pathway.

where the Ap neurons are driven by Robo2 expression. It is sometimes difficult to determine which pathway the dMP2 and vMP2 axons are in (i.e., intermediate vs. lateral) because the two pathways intertwine in these gain-of-function embryos. Comparing the two experiments, Robo2 drives the Ap neurons to a non-Fas II pathway, while it drives dMP2 and vMP2 (which normally follow the medial Fas II pathway) into either the intermediate or lateral Fas II pathway.

Interestingly, of all of the GAL4 lines we have tested (J. H. S., unpublished results), this is the one line in which we can alter lateral position by increasing the expression of Robo (Figure 8). When Robo is expressed at higher levels in dMP2 and vMP2, we often see the axons in their normal medial Fas II pathway, but we sometimes see one or the other of these axons in the intermediate Fas II pathway. The phenotype is quite variable. This exception to the rule is informative and is discussed in detail later in the Discussion.

Discussion

How do axons choose their lateral position in the developing CNS? How do axons decide which longitudinal axon pathway to follow? In this study, three lines of evidence support the model that in the *Drosophila* CNS, lateral position is determined to a large extent by the code of Robo receptors in response to a repulsive gradient of Slit. The Robo3 and Robo2 receptors are expressed on axons with different lateral boundaries. The *robo2* and *robo3* loss-of-function phenotypes shift axons medially. The *robo2* and *robo3* gain-of-function phenotypes drive axons laterally (Figure 7). We propose that growth cones express various homo- and heterodimeric combinations of Robo2, Robo3, and Robo on their surface, and that this combinatorial code of Robo receptors responds differentially to a repulsive gradient of Slit emanating from the midline.

The Robo code on its own generates a coarse topo-

graphy of projections. The Robos subdivide the CNS into approximately five broad regions from medial to lateral (defined below). The precise topography of longitudinal projections requires a combination of the Robo code with another set of cues to determine specific location. We propose that the refinement of topography is provided by discrete local cues (such as Fas II and other pathway labels). In this way, precise topography involves a simultaneous reading of both long-range and short-range guidance cues (Figure 7). Neither on its own is sufficient to generate the precision of longitudinal axon pathways. The Robo code sends axons to a particular region of the neuropil, and then local cues within that region determine precise location. It should be noted that all of our data pertain to the medial–lateral axis of the *Drosophila* CNS (probably akin to the circumferential axis of the vertebrate CNS). As yet, we know nothing about how axon position along the dorsal–ventral axis of the *Drosophila* CNS is specified (akin to the pial–luminal axis of the vertebrate CNS).

A minority of axons do not cross the midline, and they express their cell-specific combination of Robo receptors on their surface from the outset. However, the majority of axons do cross the midline, and the Robo code is not expressed on their surface until after they do so. The timing of surface expression after they cross the midline appears to be regulated posttranscriptionally and possibly controlled by cell interactions (Kidd et al., 1998a, 1998b). The Comm protein is an important component of this regulatory system (Tear et al., 1996; Kidd et al., 1998b). All CNS neurons appear to express *robo* mRNA, but the protein only appears on the axon surface at a high level after axons cross the midline. In contrast, the cell specificity of Robo3 and Robo2 expression appears to be regulated at the level of gene expression. In situ hybridization shows that the *robo3* and *robo2* mRNAs are expressed in a cell-specific fashion (Simpson et al., 2000). As with Robo, so too with Robo3 and Robo2, protein expression is temporally regulated and appears after axons cross the midline.

Evidence for the Presumptive Slit Gradient

All of these conclusions are based on the presumption of a Slit gradient emanating from the midline. We cannot directly observe this gradient, and so we do not know its shape or extent. As with most other presumptive diffusible signals in the developing organism, it is very difficult to directly show a gradient of this secreted protein in the embryo. Moreover, it has also been difficult for us to increase the slope of the gradient. Although we have been able to successfully manipulate the levels of Robo receptors throughout the embryo, and can ectopically express Slit in all neurons to generate a level playing field (Kidd et al., 1999), it has not been possible to transgenically increase the levels of Slit secreted by the midline glia. We can change the mRNA levels, but not the protein levels. Animals in which Slit is decreased—Slit heterozygous embryos or embryos carrying a hypomorphic allele—show no defects in the lateral positions of the Fas II tracts. We infer that the midline cells have some powerful mechanism of regulating their levels of secreted Slit. Experiments using transgenes expressing modified forms of Slit as attempts to alter the Slit gradient are in progress (K. S. B. and C. S. G., unpublished results).

Although it has been difficult to directly show a Slit gradient, a number of lines of evidence all support its existence in the developing embryo. The initial published immunocytochemistry with an anti-Slit mAb (Rothberg et al., 1990), and subsequent studies using the same antibody (Kidd et al., 1998a, 1998b), reveal that while most Slit remains around the midline glia, some Slit diffuses away from the midline. In particular, Slit staining is seen around longitudinal axons in the CNS neuropil, and this staining disappears in a *slit* mutant.

The existence of a Slit gradient has been functionally revealed by analysis of the migration of muscle precursors just outside of the CNS. Muscle precursors normally migrate away from the midline along the inner surface of the developing CNS; some of these muscle precursors stop just lateral to the CNS on the epidermis where they form ventral muscles. In a *slit* mutant, the mesodermal cells that form the ventral muscles fail to migrate away from the midline (Kidd et al., 1999). The same phenotype is seen in a *robo, robo2* double mutant (Sunita Kramer and J. H. S., unpublished results). Thus, mesodermal cells appear to migrate many cell diameters away from the midline by crawling down the diffusible gradient of the Slit repellent; they use both Robo and Robo2 as receptors.

In a second set of functional studies, a muscle promoter (*24B-GAL4*) was used to express a chimeric Slit receptor in these migrating muscle precursor cells. The chimeric receptor, called Robo-Fra (for Robo-Frazzled), contains the ectodomain from Robo (which binds Slit) and the cytoplasmic domain from Frazzled (the DCC homolog that sends an attractive signal) (Bashaw and Goodman, 1999). Using the GAL4 system, the chimeric receptor was turned on around the time that the muscle precursor cells have migrated laterally off the inner surface of the CNS. Once these mesodermal cells begin to express the novel receptor that detects Slit but interprets the signal as attractive, these cells turn around and migrate back toward the midline on the opposite (outside) surface of the CNS at the interface with the epidermis.

Taken together, these results strongly argue for the presence of a Slit gradient emanating from the midline. This gradient can be detected by migrating mesodermal cells on both sides of the developing CNS as far away as the lateral edge of the CNS. We reasoned that if the migrating mesodermal cells outside the CNS can detect the Slit gradient emanating from the CNS midline, then surely the navigating axons within the CNS must be able to detect the same Slit gradient within the neuropil. Moreover, we can detect Slit protein throughout the neuropil region where growth cones make their lateral positioning decisions. The loss-of-function and gain-of-function phenotypes of the various Robo family members further confirm the existence of a Slit gradient.

The Robo Code

Robo is expressed by all longitudinal axons, Robo3 is expressed at a high level by axons extending in intermediate and lateral pathways, and Robo2 is expressed at a high level by axons extending laterally. This pattern

subdivides the CNS from midline to lateral edge into three broad regions: medial, intermediate, and lateral. The detailed patterns of expression of these three receptors further subdivides these regions, making a total of at least five regions (Figures 2D and 7). Although all lateral pathways express Robo2, the most lateral pathway does not express Robo3, adding further refinement to the lateral pathways. Within the intermediate zone, the more lateral pathways express a low level of Robo2 while the more medial pathways do not.

How these different receptors respond to levels of the presumptive Slit gradient and form boundaries of expression is not known. But the data are clear; we do not see a gradient of receptor expression, but rather we see step functions in levels of expression and clean boundaries of well-defined regions. This is in some ways reminiscent of the expression of gap genes in response to the Bicoid gradient in the early fruit fly embryo. Patterning of the early embryo is largely a transcriptional problem of regulated gene expression. In contrast, patterning of axon pathways as described here is one of either the differential binding of the ectodomain of a family of receptors to a gradient of the same ligand, or the differential signaling capacity of the cytoplasmic domains of these receptors, or both. At a superficial level, the two developmental events share a similarity in their ability to form distinct regions and boundaries in response to a gradient.

The loss-of-function and gain-of-function genetic analysis of *robo2* and *robo3* supports the model whereby a combinatorial code of Robo receptors controls regional lateral position. Removal of *robo2* and/or *robo3* causes lateral axons to extend medially. This conclusion is based on the examination of two different sets of longitudinal pathways: the three major Fas II pathways and the two Connectin pathways. When *robo2* is deleted, the lateral Fas II pathway fuses with the intermediate one in a variable fashion, and in some cases the intermediate pathway fuses with the medial one. When *robo3* is deleted, the intermediate Fas II pathway fuses with the medial one. When *robo2* is deleted, the lateral Connectin pathway fuses with the medial Connectin pathway (see our related paper; Simpson et al., 2000).

When Robo2 is expressed pan-neurally, all three Fas II pathways become fused together. Pan-neural expression of Robo does not disturb lateral position in this way. But the most informative gain of-function experiments come from the use of the GAL4 system to drive cell-specific ectopic expression. Three major conclusions emerge from these experiments. First, ectopic expression of Robo2 or Robo3 on medial axons causes them to extend laterally. Second, ectopic Robo2 expression drives medial axons further laterally than does ectopic Robo3 expression. Third, ectopic Robo2 expression drives different medial axons to different lateral positions in a cell-specific fashion.

Many of these experiments were performed using the *Ap-GAL4* line, which drives expression in three Apterous neurons in each abdominal hemisegment (Figure 7). These axons normally extend medially. Ectopic Robo3 and Robo2 drive these axons laterally, whereas increased Robo does not. Ectopic Robo3 drives them laterally to just medial to the intermediate Fas II pathway. Ectopic Robo2 drives them laterally to between the intermediate and lateral Fas II pathway. In a second set of experiments, we used the *15J2 Gal4* line, which drives expression in the dMP2 and vMP2 neurons. The axons of the dMP2 and vMP2 neurons express Fas II and normally extend in the medial Fas II pathway (Figure 7). Ectopic Robo2 drives them to extend in the intermediate Fas II pathway (and sometimes the lateral Fas II pathway).

The Exception to the Rule

We argue above that Robo3 and Robo2, but not Robo, control lateral position. This conclusion is supported by the patterns of expression and the loss- and gain-of-function phenotypic analysis. However, there is one exception in which increased Robo can also alter lateral position. Of all of the GAL4 lines we have tested (J. H. S., unpublished results), the *15J2* line is the only line in which we can alter lateral position by increasing the expression of Robo (Figure 8). When Robo is expressed at higher levels in dMP2 and vMP2, we often see their axons in their normal medial Fas II pathway, but we sometimes see one or the other of these axons in the intermediate Fas II pathway. The phenotype is quite variable.

This exception to the rule is informative because these two axons are special in the spatial and temporal context of their pathway decision. Normally, the pCC growth cone pioneers the medial Fas II pathway (called the pCC pathway) as it extends anteriorly (Figure 8). The vMP2 growth cone fasciculates with it and follows right behind pCC. The MP1 growth pioneers the intermediate Fas II pathway (called the MP1 pathway) as it extends posteriorly. The dMP2 growth cone fasciculates with it and follows right behind MP1. In the middle of the segment, these four axons meet and transiently fasciculate together (Goodman and Doe, 1993; Lin et al., 1994). These axons subsequently selectively defasciculate as pCC pioneers the medial Fas II pathway, while MP1 ultimately pioneers the intermediate Fas II pathway (Hidalgo and Brand, 1997). vMP2 stays with pCC in the medial Fas II pathway. It appears as if dMP2 leaves MP1 and joins with pCC and vMP2 to join the medial pathway.

The defasciculation of these axons, and their separation to form these two distinct longitudinal pathways, occurs when *robo2* expression in all of these neurons declines; this is the same period when *robo3* appears in only MP1 of these four neurons. The medial and intermediate Fas II pathways are formed by a process of selective defasciculation, with MP1 turning on Robo3 and heading more laterally, ultimately extending in the intermediate region, while pCC continues to extend anteriorly in the medial region. In the absence of Robo3, MP1 does not defasciculate and stays with the medial Fas II pathway.

It appears as if the choice by dMP2 and vMP2 of which axon to follow—pCC or MP1—is based on a delicate balance and is exquisitely sensitive to the levels of various Robo family members (Figure 8). Increasing the level of Robo2 quite consistently tips the balance toward dMP2 and vMP2 extending more laterally with MP1 at the choice point. Increasing the level of Robo, on the other hand, has a more probabilistic effect on whether these growth cones stay with pCC (as normal) or go

with MP1. This particular choice occurs at an early stage and in a confined lateral location. It appears to be much more sensitive to the levels of Robo than does the typical choice by follower growth cones of which pre-existing longitudinal axon pathway to follow. Those more typical decisions of lateral position, such as those made by the axons of the Ap neurons, are sensitive to Robo3 and Robo2 but not to Robo. In contrast, the decision by dMP2 and vMP2 to follow either MP1's axon or pCC's axon at this Y junction choice point is also sensitive in a more variable fashion to the levels of Robo.

Precise Topography Requires More Than Just the Robo Code: Evidence for the Role of Discrete Local Cues

Robo3 and Robo2 expression define specific lateral regions. Ectopic Robo3 drives axons into the intermediate region, while ectopic Robo2 drives them even further laterally. But axons respond in a cell-specific fashion. Ectopic Robo2 drives the three Ap axons to between the intermediate and lateral Fas II pathways, while it drives the dMP2 and vMP2 axons into either the intermediate or lateral Fas II pathway (Figure 7). The control of location appears irrespective of level, since the result is consistent in spite of the different levels of expression generated by different Robo2 reporter lines, and by the variability in expression as driven with the GAL4 system (Brand and Perrimon, 1993). How can we explain this precision?

Many models for topographic specificity involve the notion of two opposing gradients, either both of the same sign (i.e., both either attractive or repulsive) in the opposite orientation (Zou et al., 2000), or both of different signs in the same orientation (e.g., Sperry, 1963; Gierer, 1987; Cheng et al., 1995; Gierer and Muller, 1995; O'Leary et al., 1999; Brown, et al., 2000). Such models are very attractive to explain certain aspects of sensory maps in the brain.

However, such models need not apply to all topographic projections. Thus far, we have found no evidence for a second gradient working in concert with the repulsive Slit gradient. We have tested the theory that an attractive Netrin gradient might be the opposing force. We have examined a variety of loss-of-function and gain-of-function conditions using genetic reagents that alter either the ligand (Netrin) or its receptor (Frazzled/DCC). In neither case do we find any evidence that the Netrin gradient plays a major role in the control of lateral position (Theresa Ho and J. H. S., unpublished results).

If there was a second opposing gradient, we might expect to see some evidence for it in the resulting phenotypes when we add or subtract Robo receptors, but we do not. While none of these observations disproves the existence of an opposing gradient, taken together, they do raise the possibility that some other model might be more appropriate.

The most parsimonious model is that precise topography in the medial–lateral axis of the *Drosophila* CNS requires two opposing forces: long-range repulsion and short-range attraction (Figure 7). Cues might exist, for example, that mark the boundary of the neuropil. But in terms of location with the neuropil, all that is required is an opposing force to the Slit gradient—it need not be a long-range gradient itself. Discrete local cues would be sufficient. Clearly, the long-range repulsion is controlled by the Slit gradient and the Robo code. We propose that the opposing force is short-range attraction as controlled by discrete local cues, one of which is Fasciclin II. In this way, the Robo code specifies the lateral region, while local cues specify precise location within that region.

The strongest support of this model involves the specification of the three major Fas II pathways (Figure 7). Fas II is a homophilic cell adhesion molecule expressed on axons that fasciculate together in three major longitudinal pathways: one medial, one intermediate, and one lateral. Growth cones expressing Fas II and Robo pick the medial Fas II pathway. Growth cones expressing Fas II, Robo3, and Robo pick the intermediate Fas II pathway. Presumably, the attraction of the medial Fas II pathway is insufficient to balance the repulsion mediated by Robo3. Growth cones expressing Fas II, Robo2, Robo3, and Robo pick the lateral Fas II pathway. In this case, it is not until they contact the lateral Fas II pathway that the Fas II-mediated attraction is stronger than the Robo2-mediated repulsion. Removal of Robo3 leads to only two Fas II pathways in which the intermediate pathway is missing, and instead the medial pathway is twice as thick. Ectopic expression of Robo2 in the dMP2 and vMP2 neurons, which normally extend in the medial Fas II pathway, drives their axons into either the intermediate or lateral Fas II pathway. Specificity is determined by the combination of Fas II and the particular Robo family members (Figure 7).

We propose that other pathways are specified by other pathway labels. For example, two pathways—one medial and the other lateral—express Connectin, another homophilic cell adhesion molecule. Growth cones expressing Connectin and Robo pick the medial Connectin pathway, while growth cones expressing Connectin and Robo2 (and presumably Robo and Robo3) pick the lateral Connectin pathway. Removal of Robo2 leads to only one fused medial Connectin pathway.

How Do Robos Read and Respond to the Slit Gradient?

How are Robo3 and Robo2 different from Robo? How do Robo3 and Robo2 specify lateral position? Why does Robo3 drive axons into the intermediate region, while Robo2 drives them into the lateral region? Robo3 and Robo2 must differ from one another in either their ectodomains (and thus have different abilities to read the Slit gradient), or in their cytoplasmic domains (and thus have different abilities to signal), or both. What are the key differences that allow them to drive axons to different lateral regions? Both of these receptors (Robo3 and Robo2) differ from Robo in some quality of their signaling, either having some additional output or missing some output found in Robo. Their cytoplasmic domains are quite different from Robo, but what differences are key for determining lateral position?

The Dickson and Goodman laboratories are currently collaborating to determine what differences amongst the Robos allow the Robo code to specify lateral position. It will be of interest to determine to what extent

different chimeric receptors and mutated receptors can drive lateralization. Preliminary collaborative results suggests that it should be possible to separate the functions of the various ectodomains and cytoplasmic domains.

Experimental Procedures

Protein Immunocytochemistry

Immunocytochemistry was done as described in our related paper (Simpson et al., 2000), with the following modifications. For HRP staining, the following concentrations were used: Robo mAb 13C9 1:10; Robo2 polyclonals 1:500–1:1000; Robo3 mAb 14C9 1:10; 1D4 1:5; BP102 1:10. Secondary antibodies were obtained from Jackson. For amplification of anti-βGal staining, Vectastain Elite ABC kit was used. For fluorescent staining, the following dilutions were used: BP102 1:20: Cappel rabbit anti-βgal 1:10,000; Alexa-488 gt anti-rabbit 1:4000 (Molecular Probes); Cy3 gt anti-mouse 1::2000 (Jackson Labs). Directly conjugated Ms anti-HRP (gift of J. Thomas) was used at 1:500.

Generation of Antibodies

Six-histidine-tagged fusion proteins were constructed by subcloning PCR-amplified fragments into the Qiagen pQE30 vector series. Robo2 antibodies were generated to an antigen of 33 kDa encompassing the 2nd, 3rd, and 4th Ig domains. Robo3 antigens of 48 kDa (Ig4 and 5 and Fn 2 and 3: amino acids 317–732) and 36 kDa (cytoplasmic: amino acids 1008–1327) resulted in antibodies showing identical patterns. The antigens were purified under denaturing conditions, dialyzed against PBS, and injected into mice in Ribi adjuvent. Monoclonal antibodies were made as previously described (Kidd et al., 1998a).

Electron Microscopy

Stage 16 Canton S embryos were hand devitellinized, opened dorsally, and prepared for immunoelectron microscopy according to procedures described previously (Lin et al., 1994) with the following modifications: following PLP fixation, the embryos were rinsed with Na-cacodylate buffer and fixed an additional 10 min with 0.025–0.05% glutaraldehyde in Na-cacodylate buffer. The fixed embryos were incubated sequentially with mouse anti-serum against Robo2 (1:500), mAb 13C9 against Robo (1:1), or mAb 14C9 (1:1) against Robo3 overnight at 4°C, biotinylated goat anti-mouse secondary antibody (1:100) for 1 hr at room temperature, and then streptavidin-conjugated HRP (1:100) for 1 hr at room temperature.

Genetics

pUAS-robo3 was assembled in two steps: a NotI 5′ fragment was first cloned into pUAST, followed by a NotI-XbaI fragment. Transformants were generated and RNAi injections were performed using standard procedures. Subclones of 1–2 kb pieces of the ectodomains of Robo2 and Robo3 were used to make sense and antisense RNA, which were annealed to make double-stranded RNA for injection. dsRNA was injected into mutant stocks balanced over CyoWg-βgal so that the mutants could be identified after injection by staining with anti-βgal. Overexpression of Robo2 was done using an *elav-GAL4 3A* line with the ElaV Enhancer and GAL4 hopped onto the 3rd chromosome generated by A. DiAntonio. The *UAS-roboY-F* transgene is from Bashaw et al., 2000 and the *UAS-robo* transgenic lines are from Kidd et al., 1998a. *Comm*$^{\Delta e39}$ is a published null allele. Overexpression in neural subsets was done by crossing *Ap-GAL4* (J. Thomas) or *15J2-GAL4* (Hidalgo and Brand, 1997) to *UAS-tlacZ*, *UAS-robo*; *UAS-robo2*; *UAS-robo3*; and EP2582 (which drives Robo2,) respectively. This allowed the scaffold to be visualized with a mouse antibody BP102 or mAb 1D4 and the Apterous or vMP2, dMP2 axons to be marked with a Rabbit anti-βgal. The generation of *UAS-robo2* and *UAS-robo3* was described in Simpson et al., 2000. The EP2582 line containing a P element upstream of Robo2 was part of the initial Rorth collection (1996) and was mapped by inverse PCR and sequencing; its location was later confirmed by the BDGP sequencing effort.

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